ATTACHMENT A

Attached is a copy of Ingber et al., *Proc. Natl. Acad. Sci.USA*, **78(6)**:3901-3905 (1981), which is referenced in the Amendment.

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Role of basal lamina in neoplastic disorganization of tissue architecture

(basement membrane/epithelial cell polarity/cell shape/immunofluorescence)

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ABSTRACT We have studied a transplantable carcinoma of the rat pancreas [Reddy, J. K. & Rao, M. S. (1977) *Science* 198, 78–80] that is composed of cytologically differentiated acinar cells that have lost their epithelial orientation and do not form acini. Light microscopy shows, however, consistent palisading, reorientation, and polarization of these cells in areas of contact with the vasculature. Electron microscopy reveals a normal basal lamina (BL) along the basal portions of repolarized tumor cells that is physically separate from the endothelial BL. We used indirect immunofluorescence to examine the distribution of BL constituents, laminin (Ln) and type IV collagen (type IV), within the different microenvironments of this tumor. In normal pancreas, Ln and type IV are distributed linearly, outlining acini and blood vessels. In the tumor parenchyma, type IV is not detected, whereas Ln appears in a punctate distribution outlining cells. Reorientation of tumor cells is observed only along linearly deposited Ln and type IV bordering vessels. These data indicate that this nonmetastatic tumor has lost the ability to produce or maintain a complete BL within its disorganized parenchyma, while its cells retain the capacity to produce and reorganize along linear BL when in contact with vascular adventitia. We suggest that failure to maintain a complete BL may be involved in the neoplastic disorganization of normal tissue architecture as well as in the breakdown of boundaries during the development of invasive carcinomata.

Cells in a epithelium are highly organized and commonly exhibit polarized form as well as function. The cells always sit on top of a continuous basement membrane[†] or basal lamina (BL) and are physically separated from the underlying connective tissue. A normal adult epithelium is a dynamic structure (1), and its orderly renewal (e.g., wound healing) requires the continued presence of BL as an extracellular scaffolding or template that maintains the original architectural form and assures for accurate regeneration of preexisting structures (2).

An epithelium may escape its normally tight growth constraints and produce a less ordered arrangement or piling up of atypical epithelial cells and so result in a disorganization of epithelial form termed dysplasia. As the epithelium becomes further disorganized, it reaches the arbitrary point at which it is termed neoplastic. A histologic specimen of this tissue would be designated as carcinoma *in situ*, a premalignant condition, as long as there is no morphologic evidence of invasion through the underlying BL. Once physical penetration occurs, the neoplasm becomes invasive and is malignant, because it is now free to metastasize.

Classically, the BL has been viewed as a host barrier through which a malignant tumor must gain the ability to invade. In fact, BL is normally a specialized product of the overlying epithelial cells (3–5), which also plays a central role as a stabilizer of epithelial form and orientation in embryogenesis (3) and is maintained throughout adult life. Thus, it is possible that neoplastic disorganization of epithelial architecture as well as malignant invasion may result either from loss of maintenance of this epithelial scaffolding or through the acquisition of some new transformed cell product that compromises its structural integrity.

In order to investigate the role of BL in the maintenance of organized tissue structures as well as neoplastic disorganization, we have studied a transplantable carcinoma of the rat exocrine pancreas as a model system. This tumor is composed of cytologically differentiated acinar cells that have lost their normal epithelial organization. The tumor was discovered, with associated metastatic foci, in nafenopin-treated rats in the laboratory of Reddy and Rao (6) and was kindly provided to us for study. As the BL is a complex of different collagenous and noncollagenous macromolecules, the distribution of two ubiquitous BL constituents, the glycoprotein laminin (Ln) (7) and type IV collagen (type IV) (8), was studied in both the tumor and normal pancreas, using indirect immunofluorescence. A preliminary account of these studies has appeared in abstract form (9).

MATERIALS AND METHODS

Experimental System. Weanling male F344 Sprague–Dawley rats (Harlan–Sprague–Dawley, Madison, WI) were inoculated subcutaneously or intraperitoneally with a mechanically prepared suspension of the pancreatic acinar carcinoma in isotonic saline. All tumors used in this study were between the 18th and 23rd passage, 1–4 cm in diameter, and displayed consistent growth characteristics and morphology. In our laboratory, the tumor grows as a nonmetastatic carcinoma. Normal pancreas was obtained from either tumor-bearing animals or non-tumor-bearing animals; the distribution of Ln and type IV was identical in both.

Light Microscopy. Tissue specimens were fixed in 10% formaldehyde in phosphate-buffered saline (P_i/NaCl) and embedded in paraffin, and sections were stained with hematoxylin and eosin.

Electron Microscopy. Tumor tissue was fixed by perfusion through the left ventricle with 1% glutaraldehyde/3% (wt/vol) formaldehyde in P_i/NaCl, treated with osmium tetroxide, stained *en bloc* with uranyl acetate, and embedded in Epon/Araldite. Thin sections were stained with uranyl acetate and lead citrate (10) and were photographed on a Siemens 101 electron microscope.

Fluorescence Microscopy. Tissue was processed according to the method of Beyer *et al.* (11) except that specimens were

Abbreviations: BL, basal lamina; Ln, laminin; type IV, type IV collagen; P_i/NaCl, phosphate-buffered saline.

† "Basement membrane" is a light-microscopic term that at the level of electron microscopy refers to a basal lamina plus adjacent connective tissue matrix.

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quenched after fixation with $NaBH_4$ at 1 mg/ml in P_i/NaCl for 40 min and placed either into 2 M sucrose/P_i/NaCl or in Tissue Tek embedding medium. Tissue in sucrose was handled according to the method of Tokuyasu (12) and semithin sections (0.8–1.0 μm) were cut at −90°C on a Sorvall MT-2B ultramicrotome with a cryotomy attachment. Sections (2–4 μm) of the tissue embedded in Tissue Tek were cut at −30°C on a Damon/IEC cryostat.

Methods for preparation of our antibodies to Lm and type IV in rabbits, their characterization, and localization within BL have been published (13, 14). A fluorescein-conjugated sheep anti-rabbit immunoglobulin was used for detection of the primary antibodies (13). Control experiments with preimmune serum in the first step or fluorescein-conjugated immunoglobulin alone were consistently negative. Localizations were done on specimens from seven separate animals on seven different occasions and all findings were confirmed on cryostat sections of frozen unfixed material.

We used a Zeiss photomicroscope II equipped with phase-contrast optics and an epifluorescence illuminator containing appropriate filters for fluorescein isothiocyanate. Images were recorded on Ektachrome 400 film.

RESULTS

Light microscopy of normal rat pancreas displays the organization of exocrine cells into acini (Fig. 1*a*). Each acinar cell is typically polarized, with its nucleus being located within the basal portion of the cell while the zymogen granules fill the apical region. These cells are consistently oriented within each acinus with their bases at the periphery and apices towards the center. Thus, examination of each acinus reveals a central area filled with zymogen granules surrounding a centroacinar lumen as well as a peripheral arrangement of the basally located nuclei along the outer margin of the acinus.

On the other hand, the acinar cell tumor is characterized by a highly disorganized parenchyma with no evidence of obvious cell orientation (Fig. 1*b*). However, consistent palisading and reorientation of tumor cells can be seen in areas of direct contact with the vasculature.

Electron microscopy best displays this arrangement of cells within the tumor. Epithelial cell repolarization can clearly be seen in tumor cells that line up along the abluminal side of a tumor vessel because their nuclei are consistently located in their basal portions while the zymogen granules fill the apical regions (Fig. 2*a*). Cells in the parenchyma of the tumor, however, are highly disorganized in that the apex of one acinar tumor cell is often abnormally juxtaposed with the basal or apical portion of a neighbor. No centroacinar lumina or ductular structures have been observed. While BL is not seen between the cells within the tumor parenchyma (see also figure 4 in ref. 15), a BL with characteristic morphology is closely apposed to the basal portions of the tumor cells in areas of cell repolarization adjacent to vascular adventitia (Fig. 2*b*). The tumor BL and that of the vessel are physically separated from each other by connective tissue matrix, and both basal laminae display occasional discontinuities.

Fig. 3*a* is a phase-contrast micrograph of a semithin section of normal rat pancreas that displays acini as well as neighboring capillaries filled with erythrocytes. The distribution of type IV within this same section is characterized by linear staining outlining all acini and blood vessels (Fig. 3*b*). A phase-contrast view of the tumor (Fig. 3*c*) once again shows a disorganized

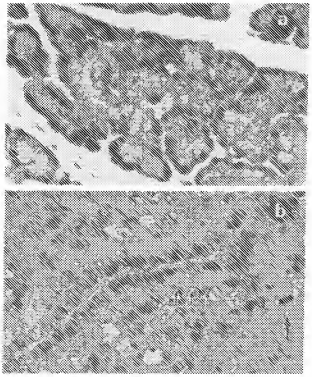

FIG. 1. Light micrographs of normal pancreas (*a*) and acinar cell tumor (*b*). (Hematoxylin and eosin; ×400.) Tips of triple arrows abut on apical poles of three neighboring reoriented tumor cells that are palisading along the adjacent vessel, larger arrow indicates a mitotic figure within the disorganized tumor parenchyma.

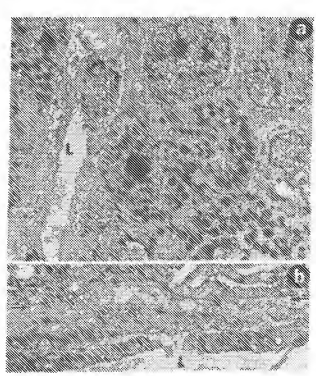

FIG. 2. (*a*) Electron micrograph of tumor containing a small blood vessel. (Uranyl acetate and lead citrate; ×5730.) (*b*) Higher magnification of the epithelial tumor–vascular interface (L, lumen of vessel; apposed arrows indicate BL underlying the basal portions of reoriented tumor cells). (×36,000.)

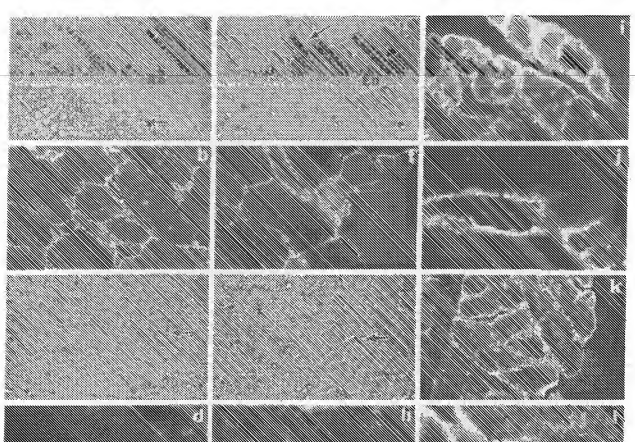

FIG. 3. Phase-contrast (*a*, *c*, *e*, and *g*) and corresponding immunofluorescence micrographs (*b*, *d*, *f*, and *h*) of semithin sections of pancreas and tumor (Ac, acinus; En, endocrine islet; arrows indicate small vessels). (*a* and *b*) Pancreas stained for type IV. (×415.) (*c* and *d*) Section of tumor stained for type IV. (×415.) (*e* and *f*) Pancreas stained for Lm. (×415.) (*g* and *h*) Tumor stained for Lm. (×415.) Immunofluorescence micrographs of standard cryostat sections of pancreas and tumor: (*i*) Pancreas stained for type IV. (×265.) (*j*) Tumor stained for type IV. (×265.) (*k*) Pancreas stained for Lm. (×265.) (*l*) Tumor stained for Lm. (×265.)

parenchyma free of acinar structures. A tumor vessel stretches horizontally across the center of the view. Under fluorescence microscopy (Fig. 3*d*) a vascular pattern of type IV staining can be seen to dominate as the tumor parenchyma is free of any obvious organized staining. This confirms the absence of any ductular or acinar forms.

Lm staining in normal pancreas (Fig. 3*f*) correlates exactly with that of type IV and once again clearly delineates all acini and vessels. Examination of the right portion of the phase-contrast view (Fig. 3*e*) reveals an endocrine islet that is characterized by linear Lm staining surrounding its capillary network. The distribution of Lm staining within a semithin section of tumor (Fig. 3*h*) is similar to that of type IV. Lm appears only in an organized linear form lining the vasculature, as can be seen in the phase-contrast view (Fig. 3*g*), and is not evident within the surrounding parenchyma.

Because semithin-sections present only a very thin slice of tissue, it was possible that our antigens were indeed present within the tumor parenchyma but in a disorganized form and were unresolvable by this technique due to presentation of an insufficient mass of antigen. To examine this possibility, thicker 2- to 4-μm conventional cryostat sections were stained for Lm and type IV as described for semithin sections.

Staining of the thicker sections of normal pancreas is once again identical for Lm and type IV (Fig. 3 *i* and *k*) and acinar forms are clearly delineated. Fig. 3*j* shows that the distribution of type IV within the tumor is identical to that seen by the semithin method with only the vascular pattern of staining evident. On the other hand, in these thicker sections Lm appears in a punctate distribution clearly outlining the tumor cells within the parenchyma as well as in a linear form along the vasculature (Fig. 3*l*).

DISCUSSION

While the pancreatic tumor does not commonly grow as an invasive carcinoma, it is an excellent model system for study of the role of BL in the organization of epithelial architecture as well as its neoplastic disorganization. Our data indicate that this rat pancreatic acinar cell tumor has lost the ability to produce or maintain a complete and organized BL within its parenchyma, and this correlates directly with loss of epithelial cell orientation. We believe that the absence of type IV staining is real

and is not due to tumor-associated changes in antigenicity. Our antibodies were developed against antigens produced by another tumor (EHS sarcoma), and antibodies produced in this manner have been shown to bind to both acid-soluble and pepsin digestion fragments of type IV collagens (unpublished data and ref. 16).

Recently a degradative enzyme has been isolated from the media of cultured metastatic tumor cells that is specific for type IV and is normally produced in a latent form that requires tryptic activation (17). The presence or absence of type IV collagenase activity or of its potential activators within the different microenvironments of this pancreatic acinar cell tumor may in part explain local differences in type IV distribution while Ln is retained. Furthermore, the punctate intercellular distribution of Ln seen in the tumor parenchyma is not unlike that seen within certain embryonic cell populations prior to their orientation into organized epithelial layers (18, 19). In the early mouse embryo, this punctate pattern of Ln is followed by deposition of type IV and subsequent organization of both molecules into linear BL (18).

It is important to emphasize that the tumor also retains the ability to organize when in direct contact with vascular adventitia. In these areas, a normal linear distribution of Ln and type IV is seen and morphologic BL appears in close apposition to the basal portions of repolarized tumor cells (Fig. 2*b*). The BL lies at the interface of the tumor cells with mesenchymally derived connective tissue that surrounds the vasculature and is physically separate from endothelial BL.

In embryological development, an epithelium usually gains the ability to produce BL as well as undergo histogenesis after having remained in direct apposition with mesenchyme throughout its characteristic induction period (20). For instance, embryonic pancreatic epithelial cells separated from their mesenchyme prior to completion of induction undergo cytodifferentiation into acinar cells, but in the absence of mitosis, production of morphologic BL, epithelial polarization, or histogenesis (21). While histogenesis depends on interactions between adjacent cellular societies, it appears that it is the BL scaffolding that serves to physically stabilize the tissue's characteristic form. Maintenance of organized morphology in salivary gland rudiments has been shown to be dependent upon the continued presence of its BL (3). The accumulation of BL by epithelia may in part be mediated through extracellular mesenchymal products such as fibrillar collagen (5, 22), although in certain systems production of new BL requires the added presence of live mesenchymally derived fibroblasts (23).

Thus, the cells of this pancreatic acinar cell tumor may be on the fulcrum of net synthesis or net breakdown of their own BL. They may be able to interact with mesenchymally derived connective tissue and, in a manner reminiscent of the embryonic state, organize and concurrently lay down a BL of their own, stabilizing this epithelial reorientation. Resultant cell polarization might redirect potential degradative activity away from the tumor cell base, further promoting accumulation of BL at the tumor margin. This hypothesis is supported by the observation that these tumor cells also organize in areas of contact with the connective tissue capsule as well as along vascular adventitia (data not shown). This finding suggests that tumor organization is not due to some vessel-specific quality such as nutrient availability and may, in part, explain the observation that this rapidly growing neoplasm does not appear to be metastatic. It is interesting to note that examination of various epithelial tumors generally reveals a direct correlation between invasive properties and the absence of BL as well as a correlation of noninvasion with the presence of continuous BL (24–26). A high correlation has also been shown between the enzymatic degradation of type IV and metastatic potential in a variety of tumor systems (27).

The lack of epithelial organization seen within the tumor parenchyma may in part be due to the tumor cells being able to survive and proliferate free of normal contact with either morphologic BL or specific BL components. Normal rat mammary epithelial cell viability appears to be dependent upon contact with an intact BL (28), and the attachment and proliferation of some other normal epithelia (29) and connective tissue cells (30) requires *de novo* collagen deposition when the cells are cultured on plastic substrata. This requirement can be circumvented by plating the epithelial cells on a layer of type IV but not type I collagen (29), and Ln appears to mediate this attachement is at least one epithelial cell line (31). Growth of various tumorigenic cell lines is, however, independent of substratum anchorage and deposition of extracellular collagen (30, 32), suggesting a correlation between loss of substratum dependence, growth autonomy, and subsequent tissue disorganization.

In addition, because the BL may function as an extracellular complex of informative or inductive molecules (20), its continued maintenance may be mandatory for normal growth regulation of organized tissues. A factor has been extracted from embryonic tissue that can replace the requirement of mesenchyme for the proliferation and cytodifferentiation of embryonic pancreatic epithelia (33). Artificial orientation of this mesenchymal factor on agarose beads leads to cell binding, cytodifferentiation, and epithelial polarization (34). If these potential mitogens were produced by mesenchyme *in vivo*, inserted into an acinar BL complex, and neutralized upon adhesion of the epithelial cell surface to the substratum, then the epithelial society should remain stable in number and form. Any process that resulted in the production or release of similar cell-associated mitogens, such as dissolution or loosening of the BL macromolecular complex, could result in the autonomous proliferation of epithelial cells.

Finally, because cell shape is tightly coupled to cell growth in normal anchorage-dependent cells (35), efficient control of cell growth within an epithelium may require a stable tissue morphology and, thus, a well-maintained BL. It is possible that a tissue's three-dimensional physical form may itself serve to regulate cell shape and orientation through transmission of the forces of tension and compression characteristic for a given architectural configuration. An epithelial structure can be regarded as a tensile or tensegrity system, that is, an architectural unit of the highest efficiency, which consists of *discontinuous* compression-resistant members (e.g., microtubules, cytoskeletal microfilaments, fibrillar collagen) interconnected directly or indirectly by a *continuous* series of tension elements (e.g., plasmalemma, contractile microfilaments, BL) (36–38). As dynamic tensile structures, cells alter their shape until an equilibrium configuration is attained that most efficiently and evenly distributes the load, given the characteristic architectural distribution of anchors within the substratum. Dissolution of BL, which frees cell anchors from their normal spatial orientation, could result in a loss of control of cell shape and thus deregulation of cell growth.

In any case, loss of type IV from the parenchyma of this pancreatic acinar cell tumor may play some role in the release of other BL constituents such as Ln from a normally organized BL as well as in the associated development of growth autonomy and loss of epithelial orientation. In other tumor systems that display both uncontrolled proliferation and invasive qualities, failure to maintain BL with additional loss of type IV at the tumor margins may be involved in the breakdown of tissue boundaries during the progression from a normal epithelium to an invasive carcinoma.

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ATTACHMENT B

Attached is a copy of Grossman, *Haematology and Blood Transfusion*, **31**: 289-298 (1987), which is referenced in the Amendment.

Haematology and Blood Transfusion Vol. 31
Modern Trends in Human Leukemia VII
Edited by Neth, Gallo, Greaves, and Kabisch

Leukemia Progression: Role of Tissue Disorganization

Z. Grossman [1]

A. Introduction

Gene expression is not fixed or irreversible. Although under normal circumstances the pattern of gene expression of specialized cells is stable and heritable, it can be altered if the regulatory circuits between nucleus and cytoplasm are modified or disrupted [1]. Thus, changes in gene expression during cell development depend not only on the nucleus, but also on the cytoplasm which plays an essential role as signal transducer. The cytoplasm, in turn, is subject to modulation by extracellular factors and via membranal interactions. This leads to the concept of "phenotypic adaptability": the capacity of cells to change their patterns of gene expression in response to changes in the microenvironment. A role for DNA methylation in stabilizing epigenetically induced changes of gene expression has been proposed [2].

The following conceptions about cancer are widely accepted: (a) cancer is caused by discrete change, or changes, in the cell genome; and (b) a series of additional mutations, in the broad sense of the word, account for the progressive evolution of the tumor phenotypes – these mutations are due to the development of genetic instability in the transformed cells [3]. In particular, nonrandom chromosome alterations have been identified in myeloid and lymphoid leukemias and lymphomas. These alterations in turn are postulated to cause changes in the expression or regulation of proto-oncogenes or other genes involved in the cell's growth and/or differentiation control. Duesberg [4] and others have questioned the validity and generality of this interpretation: it is not known whether these nonrandom chromosome changes are sufficient in themselves or even essential along with other changes for leukemogenesis; there is still no proof that activated proto-oncogenes are sufficient or even necessary to cause cancer.

On a more fundamental level, it has been argued that intracellular events cannot explain all of the changes involved in tumor progression in tissues where intercellular events regulate homeostasis [5–8]. Consistency with the concept of phenotypic adaptability, in particular, requires a more comprehensive approach. Such an approach will be outlined below, as a series of assumptions and propositions, with only a minimal reference to the supportive database (for more details and evidence, see [8–12]).

B. The Stem Cell Concept Revisited

Contrary to some theories, there is no obvious causal connection between division and differentiation-maturation at the single cell level. There is evidence in the lymphoid and hemopoietic systems and in other cell systems that differentiation can occur with or without mitosis and that the number of divisions that a cell performs at a given state of maturation is generally variable and subject to external regulation, in vivo and in vitro. Initiation of differentiation and entering into mitosis appear to be competing cellular events [13, 1]. Thus, there is no experimental

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justification for the distinction between a self-renewal or stem cell division and an amplification division, which is regarded as an intrinsic part of a maturation process. Recognizable precursor cells do appear to divide and mature simultaneously under most conditions. However, this does not imply a constitutive relationship; it may reflect only a high rate of cycling, a high relative rate of maturation, and possibly an overlap between posttranscriptional and cell-cycle processes.

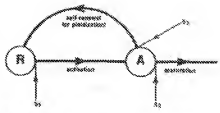

Fig. 1. Cell cycle associated levels of regulation

Assumption 1: The ratio between the probabilities of maturation and self-renewal for any mitotic cell is regulated by extracellular signals.

Corrollary: Cells other than primitive pluripotential cells have a self-renewal potential, but their self-renewal activity is tightly regulated by inter-cell interactions.

Corrollary: Competition may in principle take place not only among clones, but also within clones, i.e., among cells that belong to the same clone but are at different stages of maturation.

A number of models have been proposed on the basis of an externally regulated balance between cell division and cell differentiation-maturation [7]. A simplified possible scheme of the distinct regulatory steps in the stimulation of each cell is depicted in Fig. 1. *R* and *A* stand for "resting state" and "active state," respectively. S_1, S_2, and S_3 represent signals for initial activation, maturation, and replication, respectively. Each of these signals is partially constitutive (intracellular), partially generated (or modulated) by stromal cells, and partially elaborated by other hemopoietic cells. By definition, the latter component represents feedback. Each signal may be mediated by more than one factor or through direct intercellular interactions. There may be complete or partial overlap between the components of the different signals. Finally, the cellular origin, biochemical identity, and effects of these signals may vary according to the state of maturation of the target cell.

The dynamic aspect of hemopoiesis is provided by signals exchanged among hemopoietic cells. The feedback component need not be the main driving force, but it provides the "steering." The evidence is consistent with the notion of internal feedback circuits, operating *within* the hemopoietic tissue, as well as with peripheral signals. The large overshooting in the numbers of CFU-S and other progenitors observed following marrow treatment by irradiation or by certain drugs and the long relaxation times and cycle times in cyclic hemopoiesis can be understood if there is a delay of several days along a regulatory loop. These patterns suggest that mature or maturing hemopoietic cells control the activity of earlier progenitors. Experimental evidence implicates granulocytes, monocytes, and lymphoid cells in this function.

Assumption 2: Maturation pressures on all progenitors and precursors increase with the size of the mature cell compartment.

In Fig. 2, Xi and Yi are resting and active cells in the i-th compartment, respectively ($i=1,\ldots n$); Z are mature cells. S represents the various signals indicated in Fig. 1, collectively. Although it is convenient to think of S as representing a set of factors, the feedback effects could be exerted more indirectly. Mature cells could modulate signals exchanged between progenitors or delivered to them by stromal cells. Alternatively, mature cells could interfere with autocatalytic proliferative signals exchanged among physically adjacent progenitors.

This minimal feedback model is based on the fact that differentiation in itself is growth-limiting. Differentiation out of a compartment decreases the population remaining within; no other inhibitory force is required. It has been assumed that differentiation pressures increase with the size of the system. The cells belonging to more primitive compartments are less sensitive to these pressures than their more differentiated progeny. Resistance to differentiation

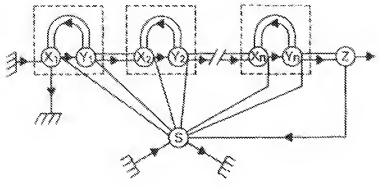

Fig. 2. "Balance-of-growth" model of hemopoiesis

pressures is a measure of the cell's self-renewal capacity. Thus, at steady-state the probabilities of self-renewal and differentiation in the earliest (most primitive) compartment are dynamically adjusted to be equal ($P=0.5$ each). The "amplification" seen in the whole system results from renewal at probability smaller than 0.5 per activation event. It can rise to higher values when the steady state is perturbed following a change in peripheral demand, a tissue insult, or under culture conditions. Indeed, there is growing evidence that essentially normal cells of the committed progenitor phenotype can exhibit extensive self-renewal in vitro, in Dexter cultures or in suspension.

This scheme is largely nonspecific, embodying the view that the hemopoietic tissue functions as an integrated system rather than a set of cell lineages developing in parallel. This view is consistent with the data on growth factors active in cell culture which suggest that each of these factors effects a variety of progenitor cells at the earlier stages of differentiation and only later do lineage-specific effects become dominant (N.A. Nicola, this volume). The question, how do lineage-specific peripheral factors, such as erythropoietin or thrombopoietin, affect the production of cells of the respective lineages is discussed elsewhere [9, 10]. The scheme accounts for the fact that the response of recognizable precursor cells to changes in peripheral demand is more significant and occurs earlier than that of the colony-forming progenitors.

The rule that cells become more responsive to differentiation signals as they mature makes maturation an autocatalytic process. This, along with the feedback assumption, ensures both the stability and the flexibility of the maturation hierarchy. It explains, for instance, why the slowly cycling primitive cells are not replaced as stem cells by their more active progeny. Certain changes in an early cell or in the tissue can weaken the differentiation feedback loop, leading eventually to leukemia.

C. "Differentiation" Revisited

"Differentiation" can be defined as inheritable changes in a cell's pattern of gene expression (not necessarily irreversible). "Maturation" is a particular step(s) of differentiation regularly observed within a cell lineage when the development of the cell is well-defined and predictable. "Adaptive differentiation" is differentiation which is guided to some extent by the cell's microenvironment.

Watching a differentiation sequence cannot tell us whether it is adaptive or not. It may owe its regularity to a constant set of constraints. Introducing modifications of the environment may lead to a modified phenotypic pattern in the developing cell population, possibly due to selection rather than adaptation. Thus, single-cell experiments are necessary to test whether reprogramming of cell differentiation can be effected in the absence of selection and to compare the early developmental steps of two daughter cells subject to different conditions. Metcalf et al. have shown that two different inducers, or different concentrations of the inducer, could push daughter cells of a CFU-C into the monocytic or into the granulocytic sublineage, respectively, and the effect appeared to be inductive, not selective [14]. Such experiments are scarce. Repro-

gramming of cell differentiation clearly occurs under highly artificial conditions [1]. There is a large body of indirect evidence supporting the notion of adaptive capacity of cells during embryonic development and into adult life. The conception of cellular differentiation as partially adaptive rather than rigidly preprogrammed is more compatible with the phenotypic plasticity observed in cultured cells and during tumor progress, when the cells are subject to a substantially modified environment for a prolonged period of time.

A programmatic approach implies "lineage fidelity" and discrete, intrinsically triggered determination (commitment) events. I prefer to see commitment as a manifestation of a gradually increasing bias for a given developmental fate. Such a bias is defined by the environment as well as by characteristics of the cell, e.g., by inducers in the environment and by the corresponding receptors on the membrane of the cell. Such commitment might be modulated or even reversed under different environmental conditions. In this light, it might be of interest to assess the fate of BFU-E under conditions of erythrocytosis induced by hypertransfusion, or that of the Meg-CFC shown to increase significantly in vivo, in some animals, without accompanying thrombocytosis [15].

Both their self-renewal capacity and pluripotency are aspects of stem cells' resistance to differentiation pressures. Because the build-up of differentiation bias is slow in these cells, competing small epigenetic modifications may switch on and off or fluctuate quantitatively. On the other hand, once a significant bias is attained and differentiation starts, the bias becomes self-enhancing and eventually irreversible within the same environment. This is equivalent to "commitment."

The concept of a "lineage" is based on the premise that the differentiation pathway of normal committed cells is fixed in advance under all conditions. This also provides the rationale for many in vitro experiments which aim to take a particular subpopulation of cells out of the complex physiological environment and place them into the simpler culture environment where they could be studied in detail. The assumption is that the events observed in vitro will accurately reflect, and provide insights into, the analogous sequence of events which occur during in vivo differentiation.

In contrast, the previously discussed considerations imply that a cell is not an autonomic entity, but that its characteristics partly depend on the microenvironment and on its past developmental history [9, 16]. For example, long-term cultures provide an environment optimal for the sustained growth of cell lines and clones. Continuous proliferation may lead not only to selection of particular subpopulations but also to adaptive phenotypic changes. In some cases, such adaptation leads these cells to specialize in self-replication. A set of genes associated with division maintains a high level of expression, possibly at the expense of other sets of genes, including perhaps those responsible for the karyotypic integrity of the cell's genome. This may lead to the accumulation of chromosomal aberrations.

In this scenario cell transformation in vitro is (a) secondary to the change in the microenvironment, and (b) described as a dynamic process in which changes in DNA sequence may follow irregular "differentiative" cellular changes (i.e., heritable changes in gene expression), which in turn follow reversible epigenic effects. The considerations in this section can be summarized by the following proposition:

Proposition 1: The phenotypic patterns of hemopoietic cells, including their profile of growth characteristics, are actively regulated by the same feedback interactions which control the numerical ratios among cells. (By "phenotypic pattern" I mean the coassociation of a given set of characteristics in the same cell.)

The stage is now set for proposing scenarios of leukemia progression in vivo.

D. The Origin of CML and the Blast Crisis

As mentioned earlier, it is their lower responsiveness to maturation pressures that endows the primitive cells with a growth advantage and stably couples them to the rest of the clone in Fig. 2, in spite of their slower cycling rate. These maturation pressures on

all mitotic cells in the bone marrow are assumed to increase with the size of the mature cell compartment of that tissue.

Corollary: If the sensitivity to feedback of all the cells within a hemopoietic clone is reduced, the clone will expand in order to restore the (steady state) balance between self-renewal and differentiation.

It is convenient to define a common clonal measure of responsiveness, k, such that all cellular transition rate coefficients (or at least the maturation rates) are proportional to k, with $0 \leqq k \leqq 1$; k was named "inductivity." For normal clones $k = 1$; $k = 0$ at the limit of a complete maturation block; and cells in the intermediate range manifest different degrees of maturation arrest, in a quantitative sense.

Proposition 2. Chronic leukemia results from a reduced clonal inductivity (namely, from a partial maturation arrest).

The underlying biological mechanism could be direct (e.g., reduced numbers, or activity, of receptors for differentiation factors, or lower levels of transduction of the membranal signals) or indirect (e.g., increased numbers, or activity, of receptors for other growth factors, or impaired microenvironment). For specificity, and in line with the generally accepted interpretation, it may be supposed that a heritable change originated in an early transformed cell and propagated through the clone by proliferation and maturation of the original cell and its progeny.

What happens to normal hemopoietic cells in CML? The expansion of the leukemic clone is associated with increased maturation pressures which affect both leukemic and normal cells. At steady-state, the level of feedback control is adjusted to the reduced responsiveness of the leukemic stem cells, but is too high for normal stem cells. These are gradually induced to differentiate faster than they renew so that, in effect, normal clones become transitory.

If the feedback control mode of Fig. 2 (named "balance of growth") were the only means of controlling cell numbers, the number of mature cells at steady-state, Z_s, would be inversely related to the inductivity for $0 < k \leqq 1$. In particular, for very small k, Z_s would be very large. However, for $k = 0$, corresponding to a complete maturation block, $Z_s = 0$. This unacceptable singular behavior (in the mathematical sense) does not occur if cell density limitations are taken into account. Beyond a certain limit, increased cell density must have a negative effect on hemopoietic cell growth. This relatively nonspecific feedback suppression mode is normally secondary to the "balance of growth" mode, but becomes potentially significant in hyperplasia. Cell crowding conditions provide a selective pressure in favor of inherently fast-cycling cells; "blast cells" acquire a growth advantage over maturing cells and (slowly cycling) primitive progenitors (overriding the normal advantage of early progenitors – having stronger resistance to feedback maturation pressures).

Corollary: As the inductivity is reduced to small values, blast cells gain dominance.

Proposition 3. The blast crisis evolves from the chronic phase as a result of a progressive maturation arrest.

Note that a moderate maturation arrest accounts for the chronic phase. It is usually believed that quantitatively different types of cellular lesions are involved: while CML is associated with a proliferative abnormality, a "blast transformation" is postulated to cause "maturation block." In contrast, the present theory requires only one type of change in the function of the cell to explain both phases of the disease. The differences between CML and the blast crisis at the single-cell level may be only a quantitative one while the cell-population manifestations are drastically different. It is suggested that cell crowding, through its effect on the interactions between cells and between compartments, plays a causative role in the transition.

E. Leukemia Progression

While proposing to characterize the relevant cellular change of function – maturation arrest – and link its progression to the acquisition of malignancy, the hypothesis has yet to explain what drives this progression.

The concept of cancer progression, as defined by Foulds [3], refers to the develop-

ment of permanent, irreversible, qualitative, and heritable changes in one or more cellular characteristics. The central dogma in oncology is that these changes are due to the inherent genetic instability of the transformed cells, which in turn are manipulated by environmental selective pressures.

The present approach deviates from this dogma, or extends it, in two general aspects. First, it ascribes a deeper, more dynamic nature to the cell-environment relationship. If heritable – or at least recurrent – changes occur in somatic cells possessing extensive division capacity which affects their growth/differentiation characteristics, the tissue composition is bound to change. This in turn leads to additional cellular changes, and so on. The purpose of a more detailed analysis is to understand in quantitative terms the conditions under which the normally self-corrective, negative feedback relationship among cellular constituents may turn into a positive feedback circuit, whereby the different types of changes reinforce each other, leading to further disorganization [6, 8].

The second aspect is the suggestion that the acquisition of increased self-renewal capacity, or decreased sensitivity to maturation pressures, may reflect in the first place a *normal* adaptive capacity on the part of the transforming cells rather than aberrant genetic programs. In fact, the theoretical analysis cannot distinguish between a series of "small," frequent genetic events and a continuous (heritable) cellular change which is not associated with DNA-sequence modifications. The conditions under which the regulatory differentiation pressures are capable of preventing the accumulation of small karyotypic changes or of controlling a slow phenotypic variation may be quite similar. The difficulty to discriminate theoretically and experimentally between such genetic and epigenetic phenomena notwithstanding, it can be shown that a form of restricted adaptive variability of the cellular phenotype, beyond that which is usually implied by a "genetic program," is consistent with both the normal stability of the phenotypic patterns and their transformability in response to some perturbations. By assuming, in particular, that the growth characteristics of normal hemopoietic cells are subject to adaptive changes in both directions, and not only to down-regulation of the self-renewal capacity with differentiation, it is possible to offer explanations for the progressive nature of chronic leukemia and preleukemia. Again, cell crowding may play a causative role in the transformation process, driving differentiation in the "wrong direction."

Assumption 3: Stimulation of cells to replicate tends to induce in them (slowly) an increased capacity for self-renewal (or equivalently, reduced sensitivity to differentiation signals). Stimulation to differentiate, or mature, has the opposite effect.

Some growth factors can regulate the expression and affinity of their own receptors. Several observations indicate that the capacity for self-renewal of hemopoietic cells can be up- or down-regulated by external influences (e.g., [17, 18]) and there is indirect evidence for a role for DNA methylation [19]. When populations identified by their capacity to form colonies under certain conditions are examined by other means, striking heterogeneities are uncovered [20].

With Assumption 3 incorporated into the scheme, proposition 3 can now be translated into a mathematical model corresponding to Fig. 2, but in which the inductivities are assumed to have constitutive (fixed) components as well as variable components [8, 12]. The latter may evolve (slowly) according to the actual self-renewal activity of the cells and to the cell densities. In Fig. 3, for example, the constitutive part is chosen to be 60% of the normal value, leading to a stable (chronic) hyperplasia and a stable steady state average for the variable part. The variability in the inductivity of the blast cells which was assumed here introduced only a small correction to their average inductivity as compared to a model with no such variability; this is because the rate of change in the inductivity of these cells is small in comparison to their turnover rate. However, further computer simulations of the model demonstrated the validity of the following proposition:

Proposition 4: Under conditions of excessive cell crowding, the stability both of the growth-characteristics' profile and of the numerical balance between compartments

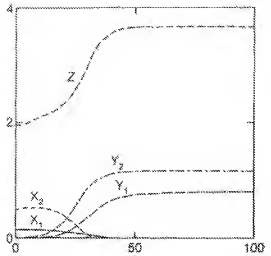

Fig. 3. Computer simulation of a simplified mathematical model corresponding to Fig. 2 with a variable inductivity for blast cells. For the stem cells, $k = 0.6$ (normally, $k = 1$). X_1 and X_2 are normal stem cell and blast cell numbers, respectively (in arbitrary units); Y_1 and Y_2 are leukemic stem cell and blast cell numbers; Z is the number of mature cells

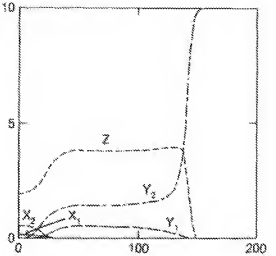

Fig. 4. Notations as in Fig. 3; $k = 0.5$

may be reversed, with a consequent dominance of a transformed subpopulation manifesting a maximal maturation arrest.

In Fig. 4, the constitutive component of the inductivity is reduced to 50% of the normal value. A transient (chronic) state of hyperplasia emerges, but a self-driven process of selection and adaptation leads to a blast crisis 100 days later. The reversal of stability results from a failure of the feedback loop – because of cell crowding – to counteract downwards fluctuations in the inductivity with (transiently) increased numbers of mature cells which would enhance the maturation pressures and up-regulate the average inductivity [12]. This failure enables such downwards fluctuations to accumulate.

Proposition 5 (Summary): An initial heritable event in an early hemopoietic cell, generating a clone with partial maturation arrest, leads to CML. If the ensuing hyperplasia distorts the interpopulation balance within the dominant leukemic clone beyond a certain level, a snowball-like process of slipping control is initiated: the distortion feeds back onto the individual members of the clone, inducing further decline in the inductivity and consequently more crowding and distortion (selection). This cascade of dynamic changes in the cells and in the tissue, which reinforce each other, leads to the blast cell dominance.

Alternative scenarios have also been proposed [8], corresponding perhaps to different pathogenic situations. In particular:

Proposition 6: Acute leukemia is a local version of the blast crisis. The whole process described above may take place in a small region of the bone marrow, and only later the dominant, transformed blast cells colonize other regions. In this case, the emergence of the acute phase is not preceded by a detectable (macroscopic) phase of chronic leukemia.

The conditions associated with the different routes of progression are not understood, but they could be related to quantitative factors that determine the order in which different cell compartments are eliminated in the course of the selection process: if the primitive leukemic progenitors are suppressed early in the process, cells that can migrate and colonize other niches may not become available until the blastic transformation is completed.

F. Dynamic Heterogeneity and "Immune Surveillance"

The leukemogenesis model described above assumed that cells in the mature compart-

ment positively affect the differentiation of earlier hemopoietic cells. In general, the feedback relationships in the tissue may include interacting cell populations which are not necessarily derived from a common stem cell. The cell populations which stimulate and regulate each other are normally linked in a stable state of "dynamic heterogeneity." A major perturbation, e.g., DNA rearrangement in a clonogenic cell, cytotoxic drug, or some kind of prolonged external stimulation, may produce a modified set of constraints, incompatible with the maintenance of dynamic heterogeneity, and malignancy may follow through a process of selection and adaptation.

It is conjectured that lymphoid cells are an important component of the regulatory cell population which generates maturation pressures in some tissue – in particular in the hemopoietic tissues. Since maintenance of these pressures is essential for the balance in the tissue, the conjecture defines a new mode of immune surveillance [11].

The old hypothesis of immune surveillance against cancer is based on two premises: (a) that transformed and normal cells generally have different antigenic qualities, and (b) that the immune system responds to the antigenically modified cells in essentially the same way as it responds to invasive microorganisms. Both premises have been questioned. Now it is suggested that a major function of lymphoid cells, in addition to their classical role as mediators of immune responses, is to assist in regulating the differentiation of a variety of normal cells. They mediate feedback interactions of the type attributed to Z in the leukemia model. The pertinent cognitive aspect of the immune systems in this capacity is recognition of self rather than recognition of foreign antigens. By forcing and steering the turnover of tissue cells, lymphoid cells do not permit accumulation of small irregular phenotypic and karyotypic changes in the tissue. Tumor escape from surveillance may be described as an escape from regulatory differentiation pressures.

The well-established association of neoplasia with various forms of immune deficiencies, the apparent enhancement of tumor "immunogenicity" as the expression of MHC antigens is increased, the role of infiltrates of lymphoid cells at tumor sites and their correlation to the stage of tumor growth and the degree of tumor differentiation [21], and the use of activated lymphoid cells in therapy – all of these should undergo reassessment under the new concept of surveillance.

G. Some Implications

Uncoupling of intracellular controls which coordinate division and differentiation in the normal cell were proposed to be the "lesion" at the root of leukemia [22, 23]. Sachs defined a hierarchy of distinct cellular changes that give rise to different phases of malignancy. While sharing with these hypotheses the concept that "arrest" is not an absolute bar to maturation, the present model associates the development of imbalance between proliferation and differentiation with uncoupling of cell subpopulations, and a progressive maturation arrest with changes in the tissue.

An animal model of transplantable leukemia [24] could serve to test these ideas. The theory predicts the possibility that blast crisis cells transplanted into healthy recipients may undergo differentiation in the host: the normal inductive forces in the host could be sufficient to induce differentiation of the partially responsive blasts and their leukemic progenitors. As the leukemic clone expands and gains dominance over the recipient hemopoietic cells a transient chronic phase may be expected to be followed by a blast crisis. Indeed, it was observed that a CML-like phase preceded the reemergence of blasts as the dominant population [24]. Chromosomal analysis is required to determine whether the mature cells at the chronic phase and the leukemic blasts belong to the same clone.

This interpretation suggests that even when reconstitution of the dominance of the normal cells is not possible (e.g., due to the depletion of the normal primitive progenitors), it may be possible to *recouple* the leukemic blast population to the rest of the leukemic clone [7] or, in other words, to restore the maturational heterogeneity. The feasibility of such a strategy depends on creating an appropriate cellular environment and pro-

viding, initially, the proper activation signals. Later on, a prolonged state of remission may be self-sustained, without therapy, if the steady state is stable (as in Fig. 3) or may require continuous intervention, at some level, if this is not the case. The period during which therapy is required may be finite if it is accompanied by adaptive cell normalization (in the model – increase in the heritable inductivity of the clonogenic cells). Due to the strong analogy drawn between the progression of acute leukemia from preleukemia and that of the blast crisis from CML, the same argument holds in principle for both diseases. As was noted [8, 12], in accord with the different respective scenarios offerred for the pathogenesis of these diseases, the dominance of blast cells in the blast crisis may be more complete and irreversible.

While chromosomal analysis in the guinea pig model has not yet been performed, new evidence in the human [25, 26] is relevant and intriguing. Recombinant DNA techniques were used to determine the origin of granulocytes in patients with acute nonlymphocytic leukemia, at presentation, in remission, and in relapse. The results provide evidence that leukemic blast cells can differentiate in vivo and that the same preleukemic clone has the potential to support normal hemopoiesis (in remission) or to allow the emergence of blastic leukemia (at presentation and in relapse). The interpretation of both the authors [25] and the editorial is that (a) leukemia arises from multiple genetic or epigenetic events, with early preleukemic stem cells coexisting with leukemic cells; (b) cytotoxic agents kill leukemic cells, but normal stem cell and preleukemic stem cells are resistant; (c) the preleukemic population can differentiate into mature elements. The experimental results, however, may be reinterpreted according to the present systemic approach. While in agreement with points (a) and (c) above, the new interpretation of the effect of the therapeutic agents is that they restore a closer-to-normal cellular environment which allows the recoupling of the blast cell compartment to the (previously suppressed) progenitor and mature cell compartments. This interpretation avoids the necessity to postulate a complete and highly selective elimination of leukemic blasts. Whatever the fate of most of the original blasts is – cell death or forced terminal differentiation – the state of remission is stabilized, at least in part, by the imposed change of cellular organization. Discrimination between these interpretations using direct observations in human patients is difficult so that studies in animal models or in Dexter's cultures are required.

Another implication of the present approach is that, since the phenotypic patterns of the transforming cells during progression are assumed to reflect the degree of their adaptation to the changing tissue conditions, a careful monitoring of these patterns may turn out to be a more reliable prognostic tool than karyotypic analysis.

H. Concluding Remarks

A single type of change in the cell function is *sufficient* to account both for CML (or preleukemia) and for the progression into the blast crisis. The *minimal* number of "events" is one. Disorganization of the tissue plays a causal role in the process, beyond a selection for more aggressive clones.

Although the theory cannot discriminate between the accumulation of heritable epigenetic effects (i.e., changes in gene expression stabilized, e.g., by DNA methylation) versus that of small modifications in the DNA sequence, the first possibility is more attractive and constitutes the "minimal" interpretation consistent with the data. The "normalcy" of the transformation process, as an expression of essentially normal cellular phenotypic adaptability, is stressed. Genetic aberrations may contribute to the process, mainly at the later stages. Note that the accumulation of phenotypic changes occurred in a transitory cell population, not in stem cells, over a period much longer than the initial turnover time of this population. Accumulation of DNA modifications is more likely to take place within self-renewing cells.

Killing or changing the behavior of all the bad cells directly might not be feasible. The present approach stresses the need for the understanding of the "dynamic heterogeneity" in the tissue in order to restore it or

prevent its disruption in the early stages of carcinogenesis or in remission.

As was recently stressed [1], current paradigms have a heavy impact on research in the field of carcinogenesis; there is a need to reevaluate the strengths and weaknesses of the presently fashionable paradigms.

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